Effects of curcumin on bleomycin-induced oxidative stress in malignant testicular germ cell tumors

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Abstract. Bleomycin is commonly used in the treatment of testicular cancer. Bleomycin generates oxygen radicals, induces the oxidative cleavage of DNA strands and induces cancer cell apoptosis. Curcumin (diferuloylmethane) is a potent antioxidant and chief component of the spice turmeric. No study investigating the effects of curcumin on intrinsic and bleomycin-induced oxidative stress in testicular germ cell tumors has been reported in the literature. For this reason, the present study aimed to examine the effects of curcumin on oxidative stress produced in wild-type NTera-2 and p53-mutant NCCIT testicular cancer cells incubated with bleomycin and the results were compared with cells treated with H_2O_2 which directly produces oxidative stress. The protein carbonyl content, thiobarbituric acid reactive substances (TBARS), glutathione (GSH), 8-isoprostane, lipid hydroperoxide (LPO) levels and total antioxidant capacity in the two testicular cancer cell lines were determined. Results showed that bleomycin and H_2O_2 significantly increased protein carbonyl, TBARS, 8-isoprostane and LPO levels in the NTera-2 and NCCIT cell lines. Bleomycin and H_2O_2 significantly decreased the antioxidant capacity and GSH levels in NTera-2 cells. Curcumin significantly decreased LPO, 8-isoprostane and protein carbonyl content, and TBARS levels increased in cells treated with bleomycin and H_2O_2. Curcumin enhanced GSH levels and the antioxidant capacity of NTera-2 cells. In conclusion, curcumin inhibits bleomycin and H_2O_2-induced oxidative stress in human testicular cancer cells.

Introduction

Testicular germ cell tumors tend to affect young males, representing the most common tumor in males aged from 20 to 40 years and the incidence has been on the increase over the last decades (1). Bleomycin has been approved by the FDA to be used alone or with other drugs as a palliative treatment of testicular cancer. Bleomycin is used in combination with etoposide and cisplatinum (BEP therapy) for the treatment of adult and childhood testicular germ cell tumors. It is known that drug combinations usually work with greater efficacy compared with monotherapy since different drugs kill cancer cells in different ways. In our study, we did not use BEP, but applied bleomycin alone as our aim was to investigate the mechanism of action of bleomycin. Bleomycin is an essential component of the cisplatin-based chemotherapy regimens used effectively in the treatment of testicular cancer (2). Bleomycin generates oxygen radicals via its ferrous binding site, and induces the oxidative cleavage of DNA strands and cancer cell apoptosis (3). Bleomycin induces a high level of oxidative stress. This is due to the unique ability of bleomycin to generate reactive oxygen species (ROS) in mitochondria. One of its effects is breaking the DNA double helix via the production of free radicals, a process that is oxygen and iron-dependent. Bleomycin forms complexes with iron that reduce molecular oxygen to superoxide and hydroxyl radicals which cause single- and double-stranded breaks in DNA. Moreover, these ROS induce lipid peroxidation, carbohydrate oxidation and alterations in prostaglandin synthesis and degradation.

Numerous in vitro studies (4-6) have demonstrated that a wide range of anticancer agents induce programmed cell death (apoptosis) in malignant cells by generating ROS which is an important therapeutic interventional approach in cancer. Oxidative stress has been shown to decrease the LD_{50} (lethal dose that kills 50% of cells) of several types of antineoplastic agents and induce cancer cell apoptosis. ROS are essential for life due to their role in numerous vital processes, including normal mitochondrial metabolism, signal transduction and the bactericidal activity of phagocytes. These molecules are formed in vivo via oxidation-reduction reactions. ROS includes free radicals, such as hydroxyl and superoxide radicals, and non-radicals, including H_2O_2 and singlet oxygen (7). Hydrogen peroxide yields the highly toxic hydroxyl radical (·OH) in the presence of reduced iron or copper via Fenton or Haber-Weiss reactions. Hydrogen peroxide easily diffuses into and out of the cells, and modulates cell proliferation, signal transduction pathways, gene expression and induces DNA damage, apoptosis and necrosis (8,9).

There is an intense debate on the concurrent use of antioxidants during conventional cancer treatments. This
argument is based on the fact that some chemotherapy drugs generate ROS which may kill cancer cells by inducing apoptosis. The induction of apoptosis via ROS is potentially an alternative mechanism for the cytotoxic effect of chemotherapeutic agents. It has been suggested that antioxidants prevent cancer cell death from ROS by inhibiting ROS and preventing ROS-induced apoptosis (7). Studies in the literature investigating the effects of various antioxidants on ROS-induced apoptosis in cancer are available (10,11). So far, only three antioxidants, NAC with cisplatinum and doxorubicin, tangeretin with tamoxifen, and β-carotene with 5-fluorouracil have been shown to decrease the effectiveness of conventional cancer therapy in vivo (12,13).

Curcumin (diferuloylmethane) is the chief component of the spice turmeric and is isolated from Curcuma longa. Curcumin is responsible for the yellow color of the spice as well as the majority of turmeric’s therapeutic effects (14). The effects of curcumin have been investigated in other cancer cell types, but not in germ cell tumors. Although curcumin has been demonstrated to have anticancer activities both in vitro in numerous cancer cell lines and in vivo models, no study has been performed investigating the effects of curcumin on oxidative stress in testicular germ cell tumors. For this reason, we studied effects of curcumin on oxidative stress in NTera-2 and NCCIT testicular cancer cells (intrinsic) incubated with curcumin alone or in combination with bleomycin, and compared these results with oxidative stress generated by incubation with H₂O₂. We determined the levels of oxidative stress markers including protein carbonyl content, thiobarbituric acid reactive substances (TBARS), glutathione (GSH), 8-isoprostane, lipid hydroperoxide (LPO) levels and total antioxidant capacity in two testicular cancer cell lines incubated with curcumin, bleomycin, bleomycin+curcumin, H₂O₂ and H₂O₂+curcumin.

Materials and methods

Cell lines. NTera-2 and NCCIT cells were obtained from the American Type Culture Collection (Manassas, VA, USA). NTera-2 and NCCIT cells were grown to confluence at 37°C in a humidified atmosphere containing 5% CO₂ in air in DMEM and RPMI medium, respectively, supplemented with 10% fetal bovine serum, 100 IU/ml penicillin and 10 µg/ml streptomycin (Invitrogen, Carlsbad, CA, USA). Results of our previous experiments (15), showed the LD₅₀ of H₂O₂ (Sigma, St. Louis, MO, USA) on NCCIT cell viability to be 35 µM, for bleomycin (Nippon Kayaku, Co., Ltd., Tokyo, Japan) as 120 µg/ml and for curcumin (Sigma) as 5 µM. Moreover, we determined LD₅₀ of curcumin on NTera-2 cell viability as 20 µM, for bleomycin as 20 µg/ml, and for H₂O₂ as 400 µM (15). We applied these doses in the incubations.

8-Isoprostane assay. As a measure of cellular oxidation, the 8-epi-prostaglandin F₂α level was measured in 100 µl samples of cell culture media using an enzyme immunoassay kit (Cat. #516351 Cayman Chemical, Ann Arbor, MI, USA) according to the manufacturer's instructions. A standard curve utilizing authentic 8-epi-prostaglandin F₂α standard was prepared and values for 8-epi-prostaglandin F₂α were reported as pg/ml of media.

TBARS assay. Lipid peroxidation was determined measuring TBARS using the TBARS assay kit (Cat. #10009055 Cayman Chemical), according to the manufacturer's instructions. Cell suspensions were centrifuged at 1,000 rpm for 5 min and washed twice with phosphate-buffered saline (PBS). Supernatants were discarded and cell pellets were resuspended in 1 ml PBS and sonicated using an ultrasonic processor three times for 5-sec intervals at the 40 V setting over ice. TBARS levels are expressed as nmol/ml.

GSH assay. Cells were scraped and collected by centrifugation. The cell pellet was resuspended in 1 ml of PBS containing 1 mM ethylenediaminetetraacetic acid (EDTA) and sonicated using an ultrasonic processor three times for 5-sec intervals at the 40 V setting over ice. The supernatant was deproteinized using 10% metaphosphoric acid (Sigma) and collected to determine total GSH levels according to the manufacturer's instructions (Cat. #703002 Cayman Chemical). The total GSH levels were expressed as nmol/mg of protein.

Protein carbonyl assay. Protein-bound carbonyl levels were measured using a protein carbonyl assay kit (Cat. #1005020 Cayman Chemical). The method was based on the covalent reaction of the carbonylated protein side chain with 2,4-dinitrophenylhydrazine (DNPH) and detection of the protein-hydrazone product at an absorbance of 370 nm. The results were calculated using the extinction coefficient of 22 M⁻¹cm⁻¹ for aliphatic hydrazones and were expressed as a nmol/mg protein.

Total antioxidant capacity assay. The total antioxidant status in cell culture lysates was determined using the commercial total antioxidant assay kit (Cat. #709001 Cayman Chemical) according to the manufacturer's instructions. The assay relies on the ability of antioxidants present in the samples to inhibit the oxidation of ABTS [2,2'-azino-di-(3-ethylbenzthiazoline sulfonate)] to ABTS⁺⁺. The amount of ABTS⁺⁺ produced is monitored by reading the absorbance at 405 nm. Total antioxidant capacity levels were expressed in mM.

LPO assay. The LPO assay measures hydroperoxides in isolated lipid-phase of samples directly following ferrous ion reduction. This assay was performed using the LPO kit (Cat. #705003 Cayman Chemical Company). LPO levels were expressed in nM.

Statistical analysis. Data were presented as the mean ± standard error. Statistical analysis was performed using SPSS packed program version 10 (SPSS Inc., Chicago, IL, USA). P<0.05 was considered to indicate a statistically significant result. Comparison of the non-parametric data among the groups was performed using the Mann-Whitney U test.

Results

8-Isoprostane levels. Following incubation with curcumin, bleomycin, bleomycin+curcumin, H₂O₂ or H₂O₂+curcumin for 72 h, cellular 8-isoPGF₂α levels were measured in NTera-2 and NCCIT cells. Incubation with bleomycin or H₂O₂ significantly increased 8-isoprostane levels in the two cell lines compared...
with the control cells and cells incubated with curcumin alone. Co-incubation of the two cell lines with bleomycin+curcumin or H\textsubscript{2}O\textsubscript{2}+curcumin decreased 8-isoprostane levels significantly compared with the cells incubated with bleomycin or H\textsubscript{2}O\textsubscript{2} alone, respectively (Fig. 1).

**TBARS levels.** TBARS levels were measured in NTera-2 and NCCIT cells following incubation with curcumin, bleomycin, bleomycin+curcumin, H\textsubscript{2}O\textsubscript{2} or H\textsubscript{2}O\textsubscript{2}+curcumin for 72 h. TBARS levels were significantly increased in bleomycin and H\textsubscript{2}O\textsubscript{2}-treated groups when compared with the control cells. No significant difference was observed in TBARS levels measured in cells incubated with curcumin when compared with the control cells. Co-incubation of cells treated with bleomycin+curcumin or H\textsubscript{2}O\textsubscript{2}+curcumin significantly reduced TBARS levels compared with the NTera-2 and NCCIT cells incubated with bleomycin or H\textsubscript{2}O\textsubscript{2} alone, respectively (Fig. 2).

**GSH levels.** Cellular GSH levels were measured in NTera-2 and NCCIT cells following incubation with curcumin, bleomycin, bleomycin+curcumin, H\textsubscript{2}O\textsubscript{2} or H\textsubscript{2}O\textsubscript{2}+curcumin for 72 h. GSH levels were not significantly different in NTera-2 cells incubated with curcumin compared with control NTera-2 cells. Bleomycin, H\textsubscript{2}O\textsubscript{2} or H\textsubscript{2}O\textsubscript{2}+curcumin significantly reduced GSH levels in NTera-2 cells compared with the control NTera-2 cells. Co-incubation with curcumin and bleomycin did not significantly increase GSH levels in NTera-2 cells compared with the NTera-2 cells incubated with bleomycin alone. GSH levels were significantly lower in NCCIT cells incubated with curcumin, bleomycin, bleomycin+curcumin, H\textsubscript{2}O\textsubscript{2} or H\textsubscript{2}O\textsubscript{2}+curcumin compared with the control NCCIT cells (Fig. 3).

**Protein carbonyl levels.** Protein carbonyl content was measured in NTera-2 and NCCIT cells following incubation with curcumin, bleomycin, bleomycin+curcumin, H\textsubscript{2}O\textsubscript{2} or H\textsubscript{2}O\textsubscript{2}+curcumin for 72 h. Curcumin decreased the protein carbonyl level significantly in NCCIT cells, but not significantly in NTera-2 cells compared with the control cells and other cell groups. The lowest significant protein carbonyl level was found in NCCIT cells incubated with curcumin. The protein carbonyl level increased in the two cell lines incubated with bleomycin or H\textsubscript{2}O\textsubscript{2}. The protein carbonyl content...
was double (600 nmol/mg) in NTera-2 cells incubated with bleomycin compared with the level (300 nmol/mg) in control NTera-2 cells. Compared with control NCCIT cells, NCCIT cells incubated with H$_2$O$_2$ showed a 3-fold increase in protein carbonyl content. Co-incubation with curcumin and bleomycin or curcumin and H$_2$O$_2$ significantly decreased protein carbonyl content in the two cell lines incubated with bleomycin or H$_2$O$_2$ alone, respectively (Fig. 4).

**Antioxidant capacity levels.** Antioxidant capacity increased significantly in NTera-2 cells incubated with curcumin in comparison with the untreated NTera-2 cells. The highest significant antioxidant capacity level was found in NTera-2 cells incubated with curcumin. By contrast, NTera-2 cells incubated with bleomycin or H$_2$O$_2$ exhibited a significant decrease in antioxidant levels compared with the control NTera-2 cells. However, co-incubation of NTera-2 cells with curcumin and bleomycin or curcumin and H$_2$O$_2$ significantly increased antioxidant levels compared with NTera-2 cells incubated with bleomycin or H$_2$O$_2$ alone, respectively. No significant difference was observed in the antioxidant levels in the NCCIT cells incubated with different agents compared with the control NCCIT cells (Fig. 5).

**LPO levels.** Curcumin treatment significantly reduced LPO levels in the two cell lines compared with the control cells. Incubation with bleomycin or H$_2$O$_2$ in the two cell lines caused a significant increase in LPO levels compared with the control cells. However, co-incubation with curcumin and bleomycin or curcumin and H$_2$O$_2$ significantly decreased LPO levels in the two cells compared with the cells incubated with bleomycin or H$_2$O$_2$ alone, respectively (Fig. 6).

**Discussion**

Curcumin is currently being evaluated as a potential chemotherapeutic agent in several clinical trials (16,17). Animal studies have shown that curcumin prevents carcinogenesis in the colon (18) and breast (19). Curcumin exhibits potent *in vitro* antiproliferative and apoptosis-inducing activities in a range of human cancer cell lines, including those derived from cancers of the prostate, breast, ovary and colon (20-22),...
but no study has been carried out with regard to the effects of curcumin on oxidative stress in testicular cancer cells.

We used a curcumin concentration in our experiments relevant to the antioxidant (curcumin) uptake concentration in humans. There are conflicting studies in the literature with regard to the oral intake of curcumin and its serum and urine concentrations. Evidence suggests that orally administered curcumin accumulates in gastrointestinal tissues. For instance, when colorectal cancer patients were administered 3.6 g/day of curcumin orally for seven days prior to surgery, curcumin was detected in malignant and normal colorectal tissue (23). By contrast, curcumin was not detected in the liver tissue of patients with liver metastases of colorectal cancer following the same oral dose of curcumin (24), suggesting that oral curcumin administration may not effectively deliver curcumin to tissues outside the gastrointestinal tract. Results of a clinical study (25) have shown that no curcumin was detected in the serum of participants administered with 500; 1,000; 2,000; 4,000; 6,000 or 8,000 mg curcumin. The presence of curcumin was only detected in two subjects (one receiving 10,000 mg and one receiving 12,000 mg). No plasma concentrations of curcumin were detected in the remaining subjects receiving 10,000 or 12,000 mg dose levels. By contrast, in a clinical trial conducted in Taiwan, the serum concentration of curcumin was usually found to peak at 1-2 h following oral intake of curcumin and gradually declined within 12 h (26). The average peak serum concentrations following the administration of 4,000; 6,000 and 8,000 mg curcumin were 0.51±0.11, 0.63±0.06 and 1.77±1.87 µM, respectively. Urinary excretion of curcumin was undetectable. We incubated two types of testicular cancer cells with various concentrations of curcumin and selected the doses of 20 µM for NTera-2 cells and 5 µM for NCCIT cells. These doses were higher than the doses used in the study in Taiwan (26). The reason for using higher curcumin doses in our study was that other investigators (25) did not detect curcumin in serum with the same doses used by the investigators in Taiwan (26). To eliminate these conflicting results, we selected effective curcumin concentrations based on our previous experimental results (15).
formation of ROS with ultimate progression to lipid peroxidation (27). This effect is likely a secondary event, following bleomycin-induced increase in free radical generation. Quantification of lipid peroxidation is essential to assess the role of oxidative injury in cancer. The increase in lipid peroxidation via bleomycin and the suppressive effect of curcumin were demonstrated by Venkatesan et al in rat lung injury (28).

This is the first study showing the suppressive effect of curcumin on bleomycin and H$_2$O$_2$-induced increases in LPO levels in testicular cancer cells. 8-Isoprostane is a reliable marker of oxidative stress. Bleomycin and H$_2$O$_2$ significantly increased 8-isoprostane levels in NTERA-2 and NCCIT cells. Increases in 8-isopGF$_2$O levels as a function of bleomycin and H$_2$O$_2$ exposure have not been previously reported in testicular cancer cells. Co-incubation with curcumin significantly decreased 8-isoprostane levels in the two cell lines incubated with bleomycin and H$_2$O$_2$. Similar to our findings, curcumin was reported to induce a decrease in 8-iso-prostaglandin levels following exposure to radiation in breast cancer cells (29).

The measurement of TBARS is a well-established method for screening and monitoring lipid peroxidation. In the present study, we demonstrated that bleomycin and H$_2$O$_2$ increased TBARS levels in the two cell lines compared with the control cells. Co-incubation of curcumin with bleomycin or H$_2$O$_2$ decreased TBARS levels compared with the cells incubated with bleomycin or H$_2$O$_2$ alone. Increased TBARS formation was reported in human hepatoma G2 cells following exposure to high levels of curcumin. By contrast, exposure to low curcumin concentration did not cause any increase in TBARS levels, similar to our findings (30).

The most general indicator and by far the most commonly used marker of protein oxidation is protein carbonyl content, based on the fact that free radicals convert amino acid side chains to carbonyl moieties in vitro. In our study, we used the method of protein 2,4-DNPH post labeling, originally introduced by Levine et al (31), for isolated proteins as a useful monitor of oxidative stress. Our data clearly demonstrate that curcumin treatment inhibits bleomycin and H$_2$O$_2$-induced protein oxidation as monitored by measuring the formation of protein reactive carbonyl contents in testicular cancer cells and provides further indication that curcumin protects cancer cells from oxidative stress via its antioxidant property. Bleomycin or H$_2$O$_2$-mediated increases in protein carbonyl content likely indicates a predisposition of testicular cancer cells to cell death. Biswas et al showed that curcumin did not have a significant effect on protein carbonyl content in arsenic carcinogenicity in humans (32). By contrast, Dance-Barnes et al reported that curcumin increased oxidative damage in mouse lung tissue by inducing protein carbonylation (33). In another study performed by Biswas et al, curcumin treatment reduced ROS generation, lipid peroxidation and protein carbonyl content, which were elevated by arsenic in Swiss albino mice (34). Bleomycin and H$_2$O$_2$ significantly decreased GSH levels in NTERA-2 cells. Bleomycin, H$_2$O$_2$, curcumin, bleomycin+curcumin or H$_2$O$_2$+curcumin led to a decrease in GSH levels in NCCIT cells. The curcumin-induced depletion of GSH was demonstrated in previous studies (35,36). Hilchey et al reported that the curcumin treatment of prostate cancer cells caused depletion of GSH. The authors reported that GSH depletion was not due to curcumin-induced ROS production (35). Curcumin-GSH interactions were demonstrated in another study performed by Awasthi et al (37). Previously, it was found that GSH S-transferase catalyzes a reaction between curcumin and GSH in Caco-2 colon cancer cells, leading to the formation of monogluthathionyl curcumin conjugates (38). The antioxidant capacity is a measure of total protective antioxidant mechanisms both for preventing the production of free radicals and for repairing oxidative damage (39). Curcumin has been shown to have beneficial effects on the antioxidant defense system, scavenge free radicals and/or prevent lipid peroxidation and it is at least 10 times more active as an antioxidant than vitamin E. In our study, bleomycin and H$_2$O$_2$ decreased total antioxidant capacity in the two testicular cancer cell lines but incubation with curcumin enhanced total antioxidant capacity. Anti-carcinogenic action of curcumin by activation of antioxidant defence system was reported in animal models and cell lines (29,40). This is the first study showing that curcumin has an inhibitory effect on bleomycin and H$_2$O$_2$-induced oxidative stress.

The probability that antioxidants interfere with the conventional cancer treatments, which are designed to prevent the mortality of cancer patients, is a complex issue. Although numerous chemotherapy drugs induce the formation of ROS, their anticancer effects do not, in general, depend on the formation of these free radicals. Antioxidant supplementation may in certain circumstances aid the prevention of free-radical-induced side effects without inhibiting the positive effects of the chemotherapy and provide a safe and effective means of enhancing the response to cancer chemotherapy. The cancer cells should divide rapidly for the cytotoxic effect of anticancer agents. Excess ROS in cancer cells slows or arrests cell growth and interferes with the effectiveness of chemotherapy since anticancer drugs are effective only when there is rapid cell proliferation. Antioxidant supplementation during chemotherapy may overcome the growth-inhibiting effects of oxidative stress and maintain responsiveness to anti-neoplastic agents (12,13). Our findings with curcumin supports these hypotheses. We found that curcumin decreases oxidative stress in germ cells induced by bleomycin, however, this does not mean that curcumin decreases the chemotherapeutic effect of bleomycin. By decreasing oxidative stress, curcumin may increase the response to bleomycin since bleomycin does not exert its chemotherapeutic action only by generating oxidative stress. Our results demonstrate the precise molecular pathways of the inhibitory effect of curcumin on oxidative stress in human testicular cancer cells induced by bleomycin. Although curcumin decreased oxidative stress in germ cells induced by bleomycin, this does not mean that curcumin decreases the chemotherapeutic effect of bleomycin. By contrast, by decreasing oxidative stress, curcumin may increase the response to bleomycin. It can be concluded that curcumin has certain inhibitory effects on oxidative stress and its concomitant use with bleomycin should be followed closely during the treatment of testicular cancer.

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References